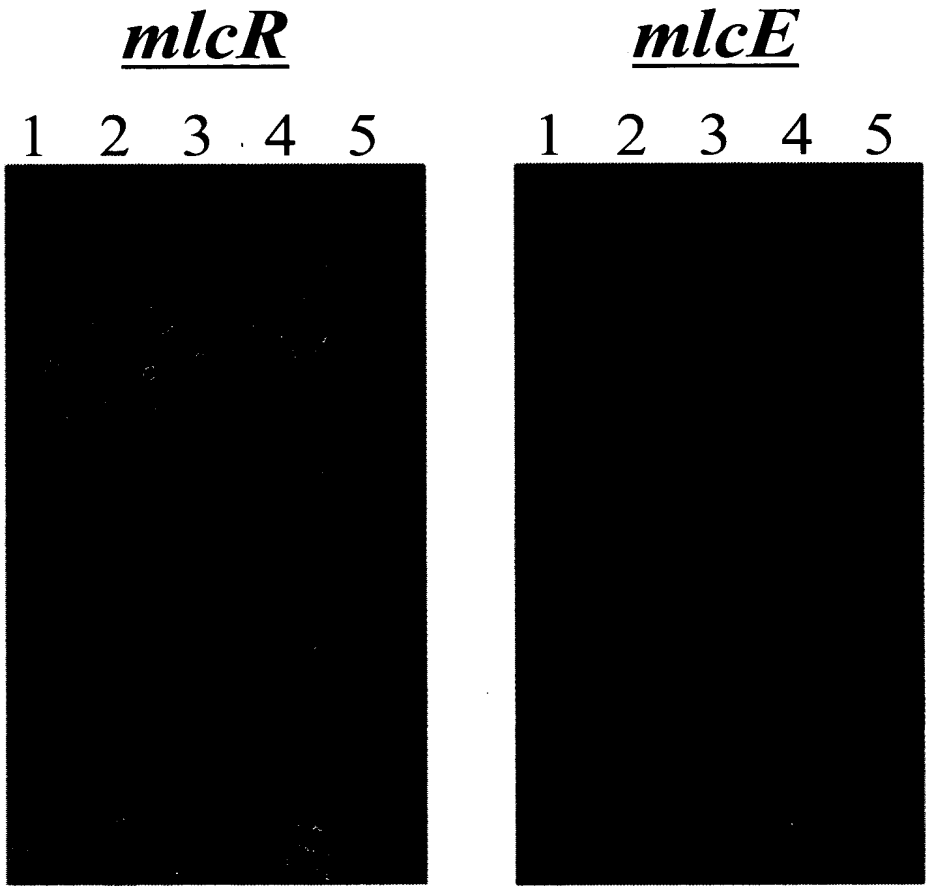

1. *Penicillium citirinum*
2. *Penicillium chrysogenum*
3. *Penicilllium notatum*
4. *Penicillium italicum*
5. *Eupenicillium sinaicum*

WO 01/12814

DESCRIPTION

DNA RELATED TO BIOSYNTHESIS OF ML-236B

[Technical Field]

The present invention relates to DNA which improves the ML-236B production ability of the producing microorganism of ML-236B, an HMG-CoA reductase inhibitor; nucleic acid molecules to be hybridized with the DNA, a recombinant vector having the DNA incorporated therein, host cells transformed by the recombinant DNA vector; preparation process of ML-236B and PCR primers designed based on the base sequence on the DNA.

[Background Art]

Pravastatin, an HMG-CoA reductase inhibitor, clinically used as a lipid lowering agent is available through the bioconversion of ML-236B produced by *Penicillium citrinum* by using *Streptomyces carbophilus* (as described in Endo, A., et al., J. Antibiot., 29, 1346(1976): Matsuoka, S., et al., Eur. J. Bioichem., 184, 707(1989)).

It is reported that ML-236B, a precursor of Pravastatin, and Lovastatin which is an HMG-CoA inhibitor sharing a common partial structure with Pravastatin are biosynthesized via a polyketide pathway (as described in Moore, R.N., et al., J. Am. Chem. Soc., 107, 3694(1985): Shiao, M. and Don, H.S., Proc. Natl. Sci. Counc. ROC., 11, 223(1987)).

Polyketide is a generic name of compounds introduced from β-keto carbon chains resulting from the successive condensation of low-molecular carboxylic acid residues such as acetic acid, propionic acid and butyric acid and it has a variety of structures, depending on the condensation reduction manner of the β-keto carbonyl group (as described in Hopwood, D.A. and Sherman, D.H., Annu. Rev. Genet., 24, 37-66(1990): Hutchinson, C.R. and Fujii, I., Annu. Rev. Genet., 49, 201-238(1995)).

Polyketide synthase (which will hereinafter be abbreviated as "PKS") taking part in the synthesis of polyketide is known to be an enzyme existing in filamentous

fungi or bacteria and molecular biological studies on this enzyme have been carried out in the filamentous fungi (as described in Feng, G.H. and Leonard, T.J., J. Bacteriol., 177, 6246(1995): Takano, Y., et al. Mol. Gen. Genet. 249, 162(1995)). In *Aspergillus terreus* which is a production microorganism of Lovastatin, a triol PKS gene has been analyzed (as described in the Japanese Patent Application Publication No. Hei 9-504436).

Genes relating to biosynthesis of secondary metabolites of filamentous fungi often form a cluster on the genome. In the biosynthesis system of polyketides, there are known to exist gene clusters participating in this system. It is known that in the biosynthesis of Aflatoxin which is a polyketide produced by *Aspergillus flavus* or *Aspergillus parasiticus*, genes encoding enzyme proteins participating in the biosynthesis of polyketides including PKS form a cluster structure, and genomic comparison and analysis of Aflatoxin biosynthesis related genes of these two microorganisms have been carried out (as described in Yu, J., et al., Appl. Environ. Microbiol., 61, 2365(1995)). It has been reported that in the biosynthesis of Sterigmatocystin produced by *Aspergillus nidulans*, biosynthesis-related genes form a cluster structure in an about 60 kb continuous region on its genome (as described in Brown, D.W., et al., Proc. Natl. Acad. Sci. USA, 93, 1418(1996)).

Molecular biological studies on the biosynthesis of ML-236B have not yet been carried out fully.

[Disclosure of the Invention]

The present inventors have found that by cloning genes or gene clusters of an enzyme taking part in the biosynthesis of ML-236B by *Penicillium citrinum* from the genomic DNA library of the ML-236B producing microorganism, and transforming the producing microorganism by using the recombinant DNA vector, the producing microorganism is able to have improved productivity of ML-236B, leading to the completion of the present invention.

The present invention relates to:

(1) DNA comprising a base sequence shown in nucleotide Nos. 1 to 34203 of SEQ ID No. 1 of the Sequence Listing, and improving the ML-236B production ability of ML-236B producing microorganism by being introduced therein,

(2) DNA as described above in (1), which is available from a transformed *E. coli* pML48 SANK71199 strain (FERM BP-6780),

(3) DNA which hybridizes with the DNA as described above in (1) or (2), and improves the ML-236B production ability of ML-236B producing microorganism by being introduced therein,

(4) DNA which hybridizes with the DNA as described above in (1) or (2) under stringent conditions, and improving the ML-236B production ability of ML-236B producing microorganism by being introduced therein,

(5) a recombinant DNA vector containing the DNA as described above in any one of (1) to (4),

(6) a recombinant DNA vector as described above in (5), which is carried by transformed *E. coli* pML48 SANK 71199 strain (FERM BP-6780),

(7) a host cell transformed by the recombinant DNA vector as described above in (5) or (6),

(8) a host cell as described above in (7) which is an ML-236B producing microorganism,

(9) a host cell as described above in (8) which is *Penicillum citrinum*,

(10) a process for preparing ML-236B, which comprises culturing the host cell as described above in (8) or (9) and then, recovering the ML-236B from the culture,

(11) a host cell as described above in (7) which is *Escherichia coli*,

(12) a host cell as described above in (11), which is a transformed *E. coli* pML48 SANK71199 (FERM BP-6780),

(13) a PCR primer A1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 23045 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(14) a PCR primer A2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer A1 as described above in (13) and having at least 10 bases (with the proviso that the PCR primer A2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 23045 to 23047 in SEQ ID No. 2 of the Sequence Listing),

(15) a PCR primer A3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer A1 as described above in (13) and having at least 10 bases (with the proviso that the PCR primer A3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 23045 to 23047 in SEQ ID No. 2 of the Sequence Listing),

(16) a PCR primer A4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer A1 as described above in (13) and having at least 10 bases (with the proviso that the PCR primer A4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 23045 to 23047 in SEQ ID No. 2 of the Sequence Listing),

(17) a PCR primer B1 comprising a sequence having at least 10 bases, with cytosine of nucleotide No. 1479 in SEQ ID No. 1 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(18) a PCR primer B2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer B1 as described above in (17) and having at least 10 bases (with the proviso that the PCR primer B2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 32720 to 32722 in SEQ ID No. 2 of the Sequence Listing),

(19) a PCR primer B3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer B1 as described above in (17) and having at least 10 bases (with the proviso that the PCR primer B3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal,

an alanine residue encoded by nucleotide Nos. 32720 to 32722 in SEQ ID No. 2 of the Sequence Listing),

(20) a PCR primer B4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer B1 as described above in (17) and having at least 10 bases (with the proviso that the PCR primer B4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 32720 to 32722 in SEQ ID No. 2 of the Sequence Listing),

(21) a PCR primer C1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 11748 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(22) a PCR primer C2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer C1 as described above in (21) and having at least 10 bases (with the proviso that the PCR primer C2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11748 to 11750 in SEQ ID No. 2 of the Sequence Listing),

(23) a PCR primer C3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer C1 as described above in (21) and having at least 10 bases (with the proviso that the PCR primer C3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11748 to 11750 in SEQ ID No. 2 of the Sequence Listing),

(24) a PCR primer C4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer C1 as described above in (21) and having at least 10 bases (with the proviso that the PCR primer C4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11748 to 11750 in SEQ ID No. 2 of the Sequence Listing),

(25) a PCR primer D1 comprising a sequence having at least 10 bases, with thymine of nucleotide No. 14362 in SEQ ID No. 1 of the Sequence Listing or a base on the 5'-side thereof

as a 5'-end,

(26) a PCR primer D2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer D1 as described above in (25) and having at least 10 bases (with the proviso that the PCR primer D2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a serine residue encoded by nucleotide Nos. 19837 to 19839 in SEQ ID No. 2 of the Sequence Listing),

(27) a PCR primer D3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer D1 as described above in (25) and having at least 10 bases (with the proviso that the PCR primer D3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a serine residue encoded by nucleotide Nos. 19837 to 19839 in SEQ ID No. 2 of the Sequence Listing),

(28) a PCR primer D4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer D1 as described above in (25) and having at least 10 bases (with the proviso that the PCR primer D4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a serine residue encoded by nucleotide Nos. 19837 to 19839 in SEQ ID No. 2 of the Sequence Listing),

(29) a PCR primer E1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 11796 in SEQ ID No. 1 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(30) a PCR primer E2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer E1 as described above in (29) and having at least 10 bases (with the proviso that the PCR primer E2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11796 to 11798 in SEQ ID No. 1 of the Sequence Listing),

(31) a PCR primer E3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer E1 as described above in (29) and having at least 10 bases (with the proviso that the PCR primer E3 is usable for PCR for

amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11796 to 11798 of SEQ ID No. 1 of the Sequence Listing),

(32) a PCR primer E4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer E1 as described above in (29) and having at least 10 bases (with the proviso that the PCR primer E4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 11796 to 11798 in SEQ ID No. 1 of the Sequence Listing),

(33) a PCR primer F1 comprising a sequence having at least 10 bases, with thymine of nucleotide No. 20723 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(34) a PCR primer F2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer F1 as described above in (33) and having at least 10 bases (with the proviso that the PCR primer F2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a cysteine residue encoded by nucleotide Nos. 13476 to 13478 in SEQ ID No. 1 of the Sequence Listing),

(35) a PCR primer F3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer F1 as described above in (33) and having at least 10 bases (with the proviso that the PCR primer F3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a cysteine residue encoded by nucleotide Nos. 13476 to 13478 in SEQ ID No. 1 of the Sequence Listing),

(36) a PCR primer F4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer F1 as described above in (33) and having at least 10 bases (with the proviso that the PCR primer F4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, a cysteine residue encoded by nucleotide Nos. 13476 to 13478 in SEQ ID No. 1 of the Sequence Listing),

(37) a PCR primer G1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 2432 | in SEQ

ID No. 1 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(38) a PCR primer G2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer G1 as described above in (37) and having at least 10 bases (with the proviso that the PCR primer G2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 24321 to 24323 in SEQ ID No. 1 of the Sequence Listing),

(39) a PCR primer G3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer G1 as described above in (37) and having at least 10 bases (with the proviso that the PCR primer G3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 24321 to 24323 in SEQ ID No. 1 of the Sequence Listing),

(40) a PCR primer G4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer G1 as described above in (37) and having at least 10 bases (with the proviso that the PCR primer G4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 24321 to 24323 in SEQ ID No. 1 of the Sequence Listing),

(41) a PCR primer H1 comprising a sequence having at least 10 bases, with thymine of nucleotide No. 6312 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(42) a PCR primer H2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer H1 as described above in (41) and having at least 10 bases (with the proviso that the PCR primer H2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an arginine residue encoded by nucleotide Nos. 27887 to 27889 in SEQ ID No. 1 of the Sequence Listing),

(43) a PCR primer H3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer H1 as described above in (41) and having at least 10 bases (with

the proviso that the PCR primer H3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an arginine residue encoded by nucleotide Nos. 27887 to 27889 in SEQ ID No. 1 of the Sequence Listing),

(44) a PCR primer H4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer H1 as described above in (41) and having at least 10 bases (with the proviso that the PCR primer H4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an arginine residue encoded by nucleotide Nos. 27887 to 27889 in SEQ ID No. 1 of the Sequence Listing),

(45) a PCR primer I1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 3545 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(46) a PCR primer I2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer I1 as described above in (45) and having at least 10 bases (with the proviso that the PCR primer I2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 3545 to 3547 in SEQ ID No. 2 of the Sequence Listing),

(47) a PCR primer I3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer I1 as described above in (45) and having at least 10 bases (with the proviso that the PCR primer I3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 3545 to 3547 in SEQ ID No. 2 of the Sequence Listing),

(48) a PCR primer I4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer I1 as described above in (45) and having at least 10 bases (with the proviso that the PCR primer I4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 3545 to 3547 in SEQ ID No. 2 of the Sequence Listing),

(49) a PCR primer J1 comprising a sequence having at

least 10 bases, with thymine of nucleotide No. 28472 in SEQ ID No. 1 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(50) a PCR primer J2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer J1 as described above in (49) and having at least 10 bases (with the proviso that the PCR primer J2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 5727 to 5729 in SEQ ID No. 2 of the Sequence Listing),

(51) a PCR primer J3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer J1 as described above in (49) and having at least 10 bases (with the proviso that the PCR primer J3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 5727 to 5729 in SEQ ID No. 2 of the Sequence Listing),

(52) a PCR primer J4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer J1 as described above in (49) and having at least 10 bases (with the proviso that the PCR primer J4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 5727 to 5729 in SEQ ID No. 2 of the Sequence Listing),

(53) a PCR primer K1 comprising a sequence having at least 10 bases, with adenine of nucleotide No. 400 in SEQ ID No. 2 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(54) a PCR primer K2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer K1 as described above in (53) and having at least 10 bases (with the proviso that the PCR primer K2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 400 to 402 in SEQ ID No. 2 of the Sequence Listing),

(55) a PCR primer K3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer K1

as described above in (53) and having at least 10 bases (with the proviso that the PCR primer K3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 400 to 402 in SEQ ID No. 2 of the Sequence Listing),

(56) a PCR primer K4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer K1 as described above in (53) and having at least 10 bases (with the proviso that the PCR primer K4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as an N terminal, a methionine residue encoded by nucleotide Nos. 400 to 402 in SEQ ID No. 2 of the Sequence Listing),

(57) a PCR primer L1 comprising a sequence having at least 10 bases, with cytosine of nucleotide No. 32287 in SEQ ID No. 1 of the Sequence Listing or a base on the 5'-side thereof as a 5'-end,

(58) a PCR primer L2 comprising a sequence having at least 70% homology with the base sequence of the PCR primer L1 as described above in (57) and having at least 10 bases (with the proviso that the PCR primer L2 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 1912 to 1914 in SEQ ID No. 2 of the Sequence Listing),

(59) a PCR primer L3 comprising a sequence having at least 80% homology with the base sequence of the PCR primer L1 as described above in (57) and having at least 10 bases (with the proviso that the PCR primer L3 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 1912 to 1914 in SEQ ID No. 2 of the Sequence Listing), and

(60) a PCR primer L4 comprising a sequence having at least 90% homology with the base sequence of the PCR primer L1 as described above in (57) and having at least 10 bases (with the proviso that the PCR primer L4 is usable for PCR for amplifying cDNA encoding a polypeptide having, as a C terminal, an alanine residue encoded by nucleotide Nos. 1912 to 1914 in SEQ ID No. 2 of the Sequence Listing).

The present invention relates to DNA originating from the genome of an ML-236B producing microorganism (which will hereinafter be called "ML-236B biosynthesis related DNA", characterized in that it improves the ML-236B production ability of the producing microorganism by being introduced therein; and the like.

The term "ML-236B producing microorganism" as used herein means a microorganism inherently having ML-236B production ability. Examples of the ML-236B producing microorganism include ML-236B producing microorganisms belonging to *Penicillium* species, more specifically, *Penicillium citrinum*, Penicillium brevicompactum (as described in Brown, A.G., et al., J. Chem. Soc. Perkin-1., 1165(1976)), *Penicillium cyclopium* (as described in Doss, S. L., et al., J. Natl. Prod., 49, 357(1986)) and the like. The other examples include *Eupenicillium* sp. M6603 (as described in Endo, A., et al., J. Antibiot. -Tokyo, 39, 1609(1986)), *Paecilomyces viridis* FERM P-6236 (as described in Japanese Patent Laid-Open No. Sho 58-98092), *Paecilomyces* sp. M2016 (as described in Endo, A., et al., J. Antibiot. -Tokyo, 39, 1609(1986)), *Trichoderma longibrachiatum* M6735 (as described in Endo, A., et al., J. Antibiot. -Tokyo, 39, 1609(1986)), *Hypomyces chrysospermus* IFO 7798 (as described in Endo, A., et al., J. Antibiot. -Tokyo, 39, 1609(1986)), *Gliocladium* sp. YJ-9515 (as described in WO9806867), *Trichoderma viride* IFO 5836 (as described in Japanese Patent Publication No. Sho 62-1915), and *Eupenicillium reticulisporum* IFO 9022 (as described in Japanese Patent Publication No. Sho 62-19159). Of these ML-236B producing microorganisms, *Penicillium citrinum* is preferred, with *Penicillium citrinum* SANK 13380 strain being more preferred. The *Penicillium citrinum* SANK 13380 strain was internationally deposited with the Research Institute of Life Science and Technology, the Agency of Industrial Science and Technology, Ministry of International Trade and Industry as of December 22, 1992 under the accession No. FERM BP-4129.

The ML-236B biosynthesis related DNA is available by screening a genomic DNA library of an ML-236B producing

microorganism with a probe designed based on a base sequence of DNA derived from a filamentous fungi which is presumed to have a similar function.

No particular limitation is imposed on method for creating the genomic DNA library provided that it is a method ordinarily employed for the creation of a genomic DNA library of an eukaryotic organism. Examples include the method of Maniatis et al. (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)).

The genomic DNA of the ML-236B producing microorganism is available by recovering the cell from the culture of the producing microorganism, physically disrupting it, and then extracting and purifying the nuclear DNA.

The ML-236B producing microorganism can be cultured under conditions suited for each of ML-236B producing microorganisms. *Penicillium citrinum*, a desirable ML-236B producing microorganism, can be cultured by inoculating the microorganism, from a slant on which the microorganism has been cultured, into an MBG3-8 medium (composition: 7% (w/v) glycerin, 3% (w/v) glucose, 1% (w/v) soybean powder, 1% (w/v) peptone (product of Kyokuto Seiyaku Kogyo Co., Ltd.), 1% (w/v) Corn steep liqueur (product of Honen corporation), 0.5% (w/v) sodium nitrate, 0.1% (w/v) magnesium sulfate heptahydrate (pH 6.5)), and keeping the temperature at 22 to 28°C for 3 to 7 days while shaking. The slant is prepared by pouring a PGA agar medium solution (composition: 200 g/L potato extract solution, 15% (w/v) glycerin, 2% (w/v) agar) into a test tube, solidifying the medium while tilting the test tube, inoculating *Penicillium citrinum* into the solidified medium by using a platinum loop, and keeping the temperature at 22 to 28°C for 7 to 15 days. Storage of the slant at 0 to 4°C enables continuous survival of the microorganisms on the slant.

ML-236B producing microorganisms cultured in a liquid medium can be recovered by centrifugal separation, while those cultured on a solid medium can be recovered by scraping them from the solid medium with a cell scraper or the like.

Physical disruption of cells can be performed by grinding the cells using a pestle and a mortar while freezing them with liquid nitrogen or the like. The nuclear DNA can be extracted from the broken cell using a surfactant such as sodium dodecylsulphate (which will hereinafter be abbreviated as "SDS"). The genomic DNA thus extracted can be recovered as a precipitate by extraction with phenol·chloroform for deproteinization and then, precipitation in ethanol.

The genomic DNA thus obtained is fragmented by restricted digestion with a suitable restriction enzyme. Although no particular limitation is imposed on the restriction enzymes to be used for the restricted digestion provided that they are ordinarily available, examples include Sau3AI and the like. The fragmented DNA is subjected to gel electrophoresis, and DNA is recovered from a gel containing a proper size of genomic DNA. Although there is no particular limitation imposed on the size of the DNA fragment, it is preferably 20 kb or greater.

Although no particular limitation is imposed on a DNA vector to be used for the construction of the genomic DNA library provided that is has a base sequence necessary for replication in the host cell transformed by the DNA vector, examples include a plasmid vector, a phage vector, a cosmid vector and a BAC vector, of which the cosmid vector is preferred. The DNA vector is preferably an expression vector. It has more preferably a base sequence which imparts selectivity of a phenotype to the host cell transformed by the DNA vector.

The DNA vector is preferably able to be used for both cloning and function expression. As such a DNA vector, a shuttle vector which can be transformed into a plurality of microorganism groups is preferably used. The shuttle vector has at least a base sequence necessary for replication in a host cell of one microorganism group. The shuttle vector preferably has a base sequence which imparts selectivity of a phenotype to each host of a plurality of microorganism groups.

Although no particular limitation is imposed on the

combination of microorganism groups to be transformed by the shuttle vector provided that one of the microorganism groups can be used for cloning and the other has ML-236B producing ability, examples include a combination of bacteria and filamentous fungi and a combination of yeast and filamentous fungi, with the combination of bacteria and filamentous fungi being preferred. Although no particular limitation is imposed on the bacteria provided that they can ordinarily be used for genetic engineering, examples include *Escherichia coli* and *Bacillus subtilis*. Of these, *Escherichia coli* is preferred, with *Escherichia coli* XL1-Blue MR strain is more preferred. As the yeast, any yeast employed ordinarily in genetic engineering is usable without particular limitation. Examples include *Saccharomyces cerevisiae*. Examples of the filamentous fungi include the above-described ML-236B producing microorganisms. In the present invention, the microorganism group can be selected from bacteria, filamentous fungi and yeast.

Examples of such a shuttle vector include cosmid vectors having a suitable marker gene for selecting a phenotype and a cos site, of which pSAKcos1 constructed by inserting a cos site of cosmid vector pWE15 (product of STRATAGENE GmbH) into plasmid pSAK333 (as described in Japanese Patent Application Publication No. Hei 3-262486) having an *Escherichia coli* hygromycin B phosphotransferase gene sequence is prefered. The shuttle vector is however not limited thereto. A method for constructing pSAKcos1 is shown in FIG. 1.

A desired genomic DNA library is completed by introducing, into a host cell, a shuttle vector in which the above-described genomic DNA fragment of ML-236B producing microorganism have been ligated. As the host cell, *Escherichia coli*, more preferably *Escherichia coli* XL1-Blue MR strain is used. When the host cell is *Escherichia coli*, introduction is performed by *in vitro* packaging. The term "transformation" as used herein means also the introduction of foreign DNA by *in vitro* packaging, and the term "transformed cell" embraces a cell into which foreign DNA has been introduced by *in vitro*

packaging.

A desired clone is screened using an antibody or a nucleic acid probe, preferably the nucleic acid probe. The nucleic acid probe can be prepared based on the base sequence of a polyketide biosynthesis related gene of a filamentous fungus. Although no particular limitation is imposed on such a gene provided that its participation in the biosynthesis of a polyketide is confirmed and the base sequence thereof is known, examples include Aflatoxin PKS genes such as *Aspergillus flavus* and *Aspergillus parasiticus*, and Sterigmatocystin PKS gene of *Aspergillus nidulans*.

The nucleic acid probe can be obtained, for example, by synthesizing an oligonucleotide probe composed of a partial base sequence of genomic DNA, by preparing an oligonucleotide primer and effecting a polymerase chain reaction (which will hereinafter be abbreviated as "PCR", as described in Saiki, R. K., et al., Science, 239, 487(1988)) with genomic DNA as a template, or by performing PCR after synthesis of cDNA using a reverse transcriptase (reverse PCR: reverse transcription-PCR which will hereinafter be abbreviated as "RT-PCR") with mRNA as a template.

A nucleic acid probe can be obtained from an ML-236B producing microorganism using PCR or RT-PCR. The primer used for PCR or RT-PCR (which will hereinafter be called "PCR primer") is designed based on the known base sequence of a polyketide biosynthesis related gene, preferably, based on the base sequence of aflatoxin PKS gene of *Aspergillus flavus* or *Aspergillus parasiticus*, or Sterigmatocystin PKS gene of *Aspergillus nidulans*. By reducing, to a base sequence, an amino acid sequence which has high interspecies conservation on the amino acid sequence of any one of these PKSs, a PCR primer can be designed. In order to reduce an amino acid sequence to the corresponding base sequence, there are two methods, that is, introduction of a single sequence in consideration of the codon using frequency of a host cell and introduction of mixed sequence (which will hereinafter be called "mix primer") using a multiple codon. In the latter

case, the multiplicity can be lowered by incorporating hypoxanthine in the base sequence.

In addition to a base sequence for annealing with a template chain, the PCR primer may be added at the 5'-end thereof a base sequence as needed. Although no particular limitation is imposed on such a base sequence provided that the resulting primer can be used for PCR, base sequences convenient for the subsequent cloning operation of a PCR product are usable. As such a base sequence, a restriction enzyme recognition sequence or a base sequence containing the restriction enzyme recognition sequence can be given as examples.

In designing of the PCR primer, the sum of the number of guanine bases and the number of cytosine bases preferably ranges from 40 to 60% of the total number of bases. Furthermore, designing which disturbs self annealing is preferred. In the case of a pair of PCR primers, designing which disturbs annealing therebetween is preferred.

Although no particular limitation is imposed on the number of bases constituting the PCR primer with the proviso that it can be used for PCR, the lower limit of the number is 10 to 14 and the upper limit is 40 to 60, with a range of from 14 to 40 being preferred.

The PCR primer is preferably DNA. Nucleosides constituting the primer include deoxyadenosine, deoxycytidine, deoxythymidine, deoxyguanosine, adenosine, cytidine, uridine and guanosine. They also include deoxyinosine and inosine.

The 5'-position of the nucleoside at the 5'-end of the PCR primer is a hydroxyl group or a hydroxy group to which monophosphoric acid has been ester bonded.

The PCR primer can be synthesized by the method ordinarily employed for the synthesis of nucleic acids, for example, the phosphoamidite method. An automated DNA synthesizer is preferably employed for such a method.

As the template for PCR and the template for RT-PCR, genomic DNA and mRNA of ML-236B producing microorganism can be used, respectively. Total RNA can also be used as the template

for RT-PCR, instead of mRNA.

The PCR product or RT-PCR product can be cloned by incorporating it in a DNA vector which is suited thereto. No particular limitation is imposed on the DNA vector used for the cloning provided that it is ordinarily employed for the cloning of a DNA fragment. Convenient kits for cloning of PCR or RT-PCR products are commercially available. For example, "Original TA Cloning Kit" (product of Invitrogen Corporation: using pCR2.1 as DNA vector) is preferably employed.

A cloned PCR product is available by culturing transformed host cells which have been confirmed to contain the desired PCR product, extracting the plasmid from the cells, and recovering the inserted DNA fragment from the purified plasmid.

The transformed host cells can be cultured under conditions suited for each host cell. Culturing of transformed *Escherichia coli* serving as a preferable host cell can be conducted by keeping it at 30 to 37°C for 18 hours to 2 days in an LB medium (1% (w/v) trypton. 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride) while shaking.

The plasmid can be prepared from the culture of the transformed host cells by recovering the host cells and removing therefrom genomic DNA and host protein. Preparation of the plasmid from the culture of transformed *Escherichia coli* serving as a preferable host cell can be carried out in accordance with the alkaline method of Maniatis (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Kits for obtaining a higher purity plasmid are commercially available and as such a kit, "Plasmid Mini Kit" (product of QIAGEN AG) is preferred for example. A kit for mass production of a plasmid is also commercially available and as such a kit, "Plasmid Maxi Kit" (product of QIAGEN AG) is preferred, for example.

The DNA concentration of the resulting plasmid can be determined by measuring absorbance of a DNA sample, which has been diluted as needed, at a wavelength of 260 nm, and

calculating on the basis that when the absorbance is 1, the concentration of DNA is 50 μg/ml. Purity of DNA can be calculated from an absorbance ratio at 260 nm and 280 nm.

Labeling of a nucleic acid probe can be roughly classified into radioactive labeling and non-radioactive labeling. Although no particular limitation is imposed on the radionuclide to be used for radioactive labeling provided that it is ordinarily employed for this purpose, examples include ^{32}P, ^{35}S, ^{14}C and the like, of which the use of ^{32}P is preferred. Although no particular limitation is imposed on the reagent to be used for non-radioactive labeling provided that it is ordinarily used for the labeling of nucleic acid, examples include digoxigenin, biotin, and the like, with digoxigenin being preferred. There is no particular imitation imposed on the method of labeling a nucleic acid probe provided that it is an ordinarily employed method. Examples of such a method include a method of incorporating in the product by PCR using a labeled substrate, the nick translation method, the random primer method, the terminal labeling method, and a method of synthesizing oligo DNA using a labeled substrate. A proper method can be selected from these methods as needed depending on the kind of the nucleic acid probe.

The presence of a base sequence of the nucleic acid probe in an ML-236B producing microorganism can be confirmed by Southern blot hybridization using the genomic DNA of the ML-236B producing microorganism.

Southern blot hybridization can be performed according to the method of Maniatis, et al (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)).

The target clone can be screened from the genomic DNA library by using the labeled nucleic acid probe prepared as described above. Although no particular limitation is imposed on the screening method insofar as it is ordinarily employed for the gene cloning, the colony hybridization method (as described in Maniatis, T., et al., Molecular Cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory,

Cold Spring Harbor, N.Y. (1989)) is usable.

A colony used for colony hybridization can be cultured under conditions suited for each host cell. Transformed *Escherichia coli* serving as a preferable host cell can be cultured by keeping it at 30 to 37°C for 18 hours to 2 days on an LB agar medium (1% (w/v) trypton, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride, 1.5 % (w/v) agarose).

Recombinant DNA vector from the positive clone available by colony hybridization is prepared by extracting the plasmid from the culture of the positive clone and purifying it.

The transformed *Escherichia coli* pML48 SANK71199 strain, a positive clone obtained by the present invention, was internationally deposited with the Research Institute of Life Science and Technology, the Agency of Industrial Science and Technology, Ministry of International Trade and Industry, as of July 7, 1999 and it was accorded accession number FERM BP-6780. The recombinant vector possessed by *E. coli* pML48 SANK71199 strain was designated as pML48.

Whether the recombinant DNA vector present in the positive clone contains a desired ML-236B biosynthesis related DNA or not can be confirmed by determination of the inserted base sequence of the recombinant DNA vector, the Southern blot hybridization or functional expression.

The base sequence of DNA can be determined by the Maxiam-Gilbert chemical modification method (as described in Maxiam, A.M.M. and Gilbert, W., Methods in Enzymology, 65, 499 (1980)), the dideoxynucleotide chain termination method (as described in Messing, J. and Vieira, J., Gene, 19, 269 (1982)) or the like. As plasmid DNA provided for determination of a base sequence, a sample having a higher purity is preferred.

The inserted base sequence of the pML48 is shown in SEQ ID No. 1 of the Sequence Listing. The base sequence shown in SEQ ID No. 2 of the Sequence Listing is completely complementary to the base sequence shown in SEQ ID No. 2. The base sequence of a genomic DNA has genetic polymorphism within its species. In the process of DNA cloning and sequencing, nucleotide substitution can occur at a certain frequency. The

present invention therefore embraces ML-236B biosynthesis related DNA which hybridizes with DNA having a base sequence shown in nucleotide Nos. 1 to 34203 of SEQ ID No. 1 or 2 of the Sequence Listing; and ML-236B biosynthesis related DNA which hybridizes, under stringent conditions, with DNA having a base sequence shown in nucleotide Nos. 1 to 34203 of SEQ ID No. 1 or 2 of the Sequence Listing. These DNAs embrace DNA having a base sequence shown in nucleotide Nos. 1 to 34203 of SEQ ID No. 1 or 2 of the Sequence Listing, wherein one or more nucleotides have been substituted, deleted and/or added; and DNA originating from ML-236B producing microorganism other than *Penicillium citrinum* SANK13380 strain, and having a function of improving the ML-236B production ability of an ML-236B producing microorganism when introduced into the ML-236B producing microorganism. The term "hybridize" as used herein means that two single-stranded nucleic acids form a double-stranded nucleic acid in a region complementary each other or in a highly complementary region, while the term "stringent condition" as used herein means the condition under which hybridization is conducted using a hybridization solution having a composition of 6 × SCC (1 × SSC has a composition of 150 mM NaCl and 15 mM of sodium tricitrate) and at a temperature kept at 55°C.

ML-236B biosynthesis related DNA is analyzed in accordance with the following methods 1) to 3).

1) Analysis by a gene analyzing software

A gene region in a genomic DNA sequence can be presumed by the existing gene analyzing program (Gene Finding program (which will hereinafter called "GRAIL") and program for searching homology of the sequence (BLASTN and BLASTX).

GRAIL is a program for searching structural genes on genomic DNA by dividing the genomic sequence into seven parameters by which "how much it resembles to the sequence of the gene" is evaluated, and integrating the results using the neural net method (as described in Uberbacher, E.C. & Mural, R.J., Proc. Natl. Acad. Sci. USA., 88, 11261(1991)). "ApoCom GRAIL Toolkit" (product of APOCOM, Inc.) is preferably

employed.

BLAST is a program using an algorithm (as described in Altechul, S.F., Madden, T.L., et al., Nucl. Acids Res., 25, 3389 (1997)) for performing homology search of nucleic acid sequences and amino acid sequences.

The position and direction of the structural gene on a sample DNA sequence can be predicted by dividing the genomic DNA sequence into suitable lengths and performing a homology search from a genetic database by using BLASTN. It is also possible to predict the position and direction of the structural gene on a sample DNA sequence by translating the divided genomic DNA sequences into amino acid sequences in accordance with 6 translation frames (three for sense sequence and the other three for the antisense sequence) and performing a homology search of the amino acid sequences using BLASTX against a peptide database. Coding regions of the structural gene in the genomic DNA are sometimes split with intron sequence in eukaryotic organisms. For analysis of the structural gene having such a gap, BLAST for gap-including sequence is more effective and Gapped-BLAST (included in BLAST2: WISCONSIN GCG package ver. 10.0) is preferred.

2) Analysis by the Northern blot hybridization method

Expression of a structural gene predicted by the analysis method described in 1) can be studied using the Northern blot hybridization method.

Total RNA of the ML-236B producing microorganism to be provided for the northern blot is available from the culture of the microorganism. *Penicillium citrinum* which is a preferable ML-236B producing microorganism can be cultured by inoculating the microorganism from the slant thereof into an MGB3-8 medium and then keeping the resulting medium at 22 to 28°C for 1 to 4 days while shaking.

Although no particular limitation is imposed on the method employed for extraction of RNA from the ML-236B producing microorganism insofar as it is ordinarily employed for the preparation of total RNA, examples include the guanidine thiocyanate hot phenol method,

guanidine·thiocyanate-guanidine hydrochloric acid method and the like. As a commercially available kit for preparing total RNA having a higher purity, "RNeasy Plant Mini Kit" (product of Qiagen AG) can be mentioned as an example. In addition, mRNA is available by adding total RNA to an oligo (dT) column, and recovering the fraction adsorbed to the column.

Transfer of RNA to a membrane, preparation of a probe, hybridization and detection of a signal can be carried out as in the above-described Southern blot hybridization method.

3) Analysis of 5'-end and 3'-end

The 5'-end and 3'-end of each structural gene can be analyzed by the RACE (rapid amplification of cDNA ends) method. The RACE is a method, by application of RT-PCR thereto, for obtaining a cDNA extending from a region having a determined base sequence to the 5'-end or 3'-end region having an undetermined base sequence, with mRNA as a template (as described in Frohman. M.A., et al., Proc. Natl. Acad. Sci. U.S.A., 85, 8988(1998)).

The 5' RACE is conducted according to the following method. After the first cDNA strand is synthesized by reverse transcription using, as a primer, an antisense-side oligo DNA (1) designed based on the known portion in the base sequence, a homopolymeric nucleotide chain (composed of a single base) is added to the 3'-end of the first strand of the cDNA by using terminal deoxynucleotidyl transferase. Then, with the first strand of the cDNA as a template, double stranded cDNA in the 5'-end region is amplified by PCR using, as primers, an sense-side oligo DNA which uses the first cDNA strand as a template and contains a base sequence complementary to the homopolymeric base sequence, and an antisense-side oligo DNA (2) existing on the 3'-end side of the oligo DNA (1) (as described in Frohman, M.A., Methods in Enzymol., 218, 340(1993)). A kit for 5' RACE is commercially available and as such a kit, 5' RACE System for Rapid Amplification of cDNA ends, Version 2.0 (product of GIBCO BRL) is preferred.

The 3' RACE is a method of making use of the polyA region existing at the 3'-end of mRNA. Described specifically,

after synthesis of a first cDNA strand by a reverse transcription using mRNA as a template and an oligo d(T) adapter as a primer, a double stranded cDNA in the 3'-end region is amplified by PCR, with the first cDNA strand as a template and with an sense-side oligo DNA (3) designed based on the known portion of the base sequence and an antisense side oligo d(T) adapter as primers. A kit for 3' RACE is commercially available and as such a kit, "Ready-To-Go T-primed First-Strand Kit" (product of Phramacia) is preferably employed.

Analytical results obtained in 1) and 2) can be preferably used for designing, in RASE, of the primer based on a known portion in the base sequence.

The analysis methods as described above in 1) to 3) enable to predict the direction of a structural gene on a genomic DNA sequence, and the position of transcription initiation point, position of a translation initiation codon, translation termination codon and position thereof in the structural gene. Based on the above-described information, each structural gene, and cDNA thereof can be obtained.

On the inserted sequence in a recombinant DNA vector pML48 obtained by the present invention, six structural genes are assumed to exist. They are named mlcA, mlcB, mlcC, mlcD, mlcE, and mlcR, respectively. Among them, mlcA, mlcB, mlcE and mlcR were predicted to have a coding region on the base sequence shown in SEQ ID No. 2 of the Sequence Listing, while mlcC and mlcD were predicted to have a coding region on the base sequence shown in SEQ ID No. 1 of the Sequence Listing.

Examples of a method of obtaining cDNA include cloning by RT-PCR using primers which can be designed in accordance with the above-described information, cloning from a cDNA library by using DNA probes available based on such information and the like.

It is necessary to obtain a full-length cDNA in order to cause functional expression of cDNA available by these methods. In addition, it is essential to design a primer so as to incorporate therein a translation initiation codon at the

original position of the RT-PCR product and so as not to incorporate, in a translation frame starting from the translation initiation codon, a translation termination codon at a position other than the original position.

PCR primer X1 (X is selected from any one of A, C, E, G, I and K and A1, C1, E1, G1, I1 and K1 are described in (13), (21), (29), (37), (45) and (53), respectively) or PCR primer Y1 (Y is selected from any one of B, D, F, H, J and L and B1, D1, F1, H1, J1 and L1 are described in (17), (25), (33), (41), (49) and (57), respectively) has a base sequence composed of at least 10 bases in the base sequence shown in SEQ ID No. 1 or SEQ ID No. 2 of the Sequence Listing.

It is not always necessary that the base sequence of the PCR primer is not completely complementary to a part of each template chain, provided that it selectively anneals with the template chain and at the same time, permits functioning as a PCR or RT-PCR primer.

PCR Primers X2 to X4 (X is selected from any one of A, C, E, G, I and K and A2, A3, A4, C2, C3, C4, E2, E3, E4, G2, G3, G4, I2, I3, I4, K2, K3 and K4 are described in (14), (15), (16), (22), (23), (24), (30), (31), (32), (38), (39), (40), (46), (47), (48), (54), (55) and (56), respectively) each comprises a base sequence having at least 70% homology, preferably at least 80% homology, more preferably at least 90% homology, with the base sequence of the PCR primer X1 (X1 and X of X2 to X4 indicate the same alphabet group; A2 to A4, C2 to C4, E2 to E4, G2 to G4, I2 to I4 and K2 to K4 correspond to A1, C1, E1, G1, I1 and K1, respectively) and has at least 10 bases.

PCR Primers Y2 to Y4 (Y is selected from any one of B, D, F, H, J and L and B2, B3, B4, D2, D3, D4, F2, F3, F4, H2, H3, H4, J2, J3, J4, L2, L3 and L4 are described in (18), (19), (20), (26), (27), (28), (34), (35), (36), (42), (43), (44), (50), (51), (52), (58), (59) and (60), respectively) each comprises a base sequence having at least 70% homology, preferably at least 80% homology, more preferably at least 90% homology, with the base sequence of the PCR primer Y1 (Y1 and

Y of Y2 to Y4 means the same alphabet group; B2 to B4, D2 to D4, F2 to F4, H2 to H4, J2 to J4 and L2 to L4 correspond to B1, D1, F1, H1, J1 and L1, respectively) and has at least 10 bases.

PCR or PT-PCR can be carried out using, as a primer, any one of PCR primers X1 to X4 (X is selected from any one of A, C, E, G, I and K) and PCR primers Y1 to Y4 (Y is selected from B, D, F, H, J and L).

As described above, 6 structural genes (m1cA, m1cB, m1cC, m1cD, m1cE and m1cR) were predicted to exist on the inserted sequence of the recombinant DNA vector pML48 obtained in the present invention. The cDNA of these 6 structural genes is available by RT-PCR in which reverse transcription and PCR using, as primers, any one of PCR primers X1 to X4 and any one of the PCR primers Y1 to Y4 are combined. The structural genes are each available by PCR using as primers any one of the PCR primers X1 to X4 and any one of the PCR primers Y1 to Y4, and, as a template, the genomic DNA of the ML-236B producing microorganism.

As a PCR primer for obtaining cDNA having a full length and capable of being expressed in a proper host cell, a combination of any one of the PCR primers X1 to X4 and any one of the PCR primers Y1 to Y4 which combination will be described next in (1) is preferably employed.

(1) A combination of any one of the PCR primers A1 to A4 and any one of the PCR primers B1 to B4 is preferably employed for acquisition of the cDNA of m1cA.

A combination of any one of the PCR primers C1 to C4 and any one of the PCR primers D1 to D4 is preferably employed for the acquisition of the cDNA of m1cB.

A combination of any one of the PCR primers E1 to E4 and any one of the PCR primers F1 to F4 is preferably employed for the acquisition of the cDNA of m1cC.

A combination of any one of the PCR primers G1 to G4 and any one of the PCR primers H1 to H4 is preferably employed for the acquisition of the cDNA of m1cD.

A combination of any one of the PCR primers I1 to I4 and any one of the PCR primers J1 to J4 is preferably employed for

the acquisition of the cDNA of m1cE.

A combination of any one of the PCR primers K1 to K4 and any one of the PCR primers L1 to L4 is preferably employed for the acquisition of the cDNA of m1cR.

Furthermore, the PCR primers X1 to X4 must satisfy the below-described requirements.

(2) The PCR primers X1 to X4 are each designed so that the PCR product obtained using any one of the PCR primers X1 to X4 and any one of PCR primers Y1 to Y4 as primers would contain a translation initiation codon atg at the original position of the RT-PCR product but would not contain, in a translation frame starting from the translation initiation codon, a translation termination codon at a position other than the original position (the position of the translation initiation codon of each structural gene predicted in the present invention, in the base sequence shown in nucleotide Nos. 1 to 34203 of SEQ ID No. 1 of the Sequence Listing and the base sequence shown in nucleotide Nos. 1 to 34203 of SEQ. ID. No. 3 of the Sequence Listing, is described in Table 4).

The PCR primer X1 has, as a 5'-end thereof, 'a' in the translation initiation codon atg, or a base existing on the 5'-end side thereof.

The PCR primers X2 to X4 selectively anneals with a specific region on the base sequence shown by nucleotide Nos. 1 to 34203 of SEQ ID No. 1 of the Sequence Listing or nucleotide Nos. 1 to 34203 of SEQ ID No. 2 of the Sequence Listing (the total base sequence of SEQ ID No. 2 of the Sequence Listing is completely complementary to the total base sequence of SEQ ID No. 1 of the Sequence Listing).

When the PCR primers X2 to X4 each contains a base sequence on the 3'-side of the translation initiation codon atg, it does not contain, on the base sequence on the 3'-side of the translation initiation codon atg, a base sequence (taa, tag or tga) which will be a termination codon in a translation frame starting from the initiation codon atg. The term "translation frame starting from an initiation codon atg" means a sequence composed of 3 bases appearing when the base

sequence on the 3'-side of the translation initiation codon atg is divided by 3 bases from the translation initiation codon atg.

In the case where the PCR primers X2 to X4 contain, at a, at or atg (which will hereinafter be called "base or base sequence m") of the translation initiation codon, a base or base sequence (which will hereinafter be called "base or base sequence m'") corresponding to the position, when the base or base sequence m is "a", the base or base sequence m' is "a" and at the same time, the "a" of the base or base sequence m' is located at the 3'-end of the PCR primers X2 to X4. Similarly, in the case where the base or base sequence m is "at", the base or base sequence m' is "at" and at the same time, "at" of the base or base sequence m' is located at the 3'-end of the PCR primers X2 to X4. In the case where the base or base sequence m is "atg", the base or base sequence m' is "atg". At the same time, when the PCR primers X2 to X4 contain a trinucleotide having, as a 5'-end, the (3×n +1)th nucleotide (n represents an integer of 1 or greater) counted from "a" of the atg of the base or base sequence m' in the direction of the 3'-end, the base sequence of the trinucleotide is none of taa, tag nor tga.

In the case where the 3'-end of the PCR primers X2 to X4 is the (3×n + 1)th nucleotide (n represents an integer of 1 or greater) counted from "a" of the atg of the translation initiation codon in the direction of the 3'-end, the base sequence of a trinucleotide composed of the (3×n + 1)th nucleotide and a dinucleotide adjacent thereto on the 3'-side thereof is none of taa, tag or tga in the RT-PCR product obtained using the PCR primers X2 to X4 as one of the primers, and RNA or mRNA of the ML-236B producing microorganism as a template, or in the PCR product obtained by using a genomic DNA or cDNA as a template.

In the case where the 3'-end of anyone of the PCR primers X2 to X4 is the (3×n + 2)th (n stands for an integer of 1 or greater) nucleotide counted from "a" of the atg of the translation initiation codon in the direction of the 3'-end,

the base sequence of a trinucleotide composed of the (3×n + 2)th nucleotide and two mononucleotides adjacent thereto on the 3'-side and 5'-side thereof is none of taa, tag or tga in the RT-PCR product obtained using the PCR primers X2 to X4 as one of the primers, and RNA or mRNA of the ML-236B producing microorganism as a template, or in the PCR product obtained by using a genomic DNA or cDNA as a template.

In the case where the 3'-end of anyone of the PCR primers X2 to X4 is the (3×n + 3)th (n stands for an integer of 1 or greater) nucleotide counted from "a" of the atg of the translation initiation codon in the direction of the 3'-end, the base sequence of a trinucleotide composed of the (3×n + 1) to (3×n + 3)th nucleotides is none of taa, tag or tga.

The above-described requirements are Requirements (2).

The following requirements (3) are for the PCR primers Y1 to Y4.

(3) The PCR primers Y1 to Y4 are each designed so that cDNA encoding the N terminal to the C terminal of a peptide encoded by each structural gene (mlcA, mlcB, mlcC, mlcD, mlcE and mlcR) can be amplified by PCR using any one of the PCR primers X1 to X4 and any one of the PCR primers Y1 to Y4 as a primers.

Although no particular limitation is imposed on the PCR primer Y1 insofar as it is a PCR primer having a base sequence complementary to a base sequence located in the vicinity of the translation termination region on the cDNA, preferred is a primer having a base sequence having, as the 5'-end thereof, a base complementary to the base at 3'-end of a translation termination codon, or a base on the 5'-end side thereof, of which a primer having a sequence of 3 bases complementary to the translation termination codon is more preferred (the translation termination codon of each structural gene predicted in the present invention, sequence complementary to the translation termination codon, an amino acid residue at C terminal of the peptide encoded by each structural gene, base sequence encoding the amino acid residue, and their positions in the base sequence shown in nucleotide Nos. 1 to 34203 of

SEQ ID No. 1 of the Sequence Listing and nucleotide Nos. 1 to 34203 of SEQ ID No. 2 of the Sequence Listing are described in Tables 8 to 10).

The PCR primers Y2 to Y4 selectively anneal with a specific region on the base sequence represented by nucleotide Nos. 1 to 34203 of SEQ ID No. 1 of the Sequence Listing or nucleotide Nos. 1 to 34203 of SEQ ID No. 2 of the Sequence Listing.

The above-described requirements are requirements (3).

In addition, it is possible to add a base sequence to the 5'-end of each of the PCR primers X2 to X4 and PCR primers Y2 to Y4 as needed insofar as the above-described definition and the above-mentioned requirements (2) and (3) are satisfied. Although no particular limitation is imposed on such a base sequence provided that the primer is usable for PCR, examples include base sequences convenient for the subsequent cloning operation of the PCR product, more specifically, restriction enzyme recognition sequences and base sequences containing such restriction enzyme recognition sequences.

The PCR primers X1 to X4 and PCR primers Y1 to Y4 are designed according to the above description concerning the design of the PCR primers.

Functional expression of the recombinant DNA vector possessed by a positive clone can be confirmed by transforming the cells by using the recombinant DNA vector and measuring the ML-236B producing ability of the transformed cells. As the cells which are used for functional expression, the above-described ML-236B producing microorganism or ML-236B non-producing microorganism is usable. No particular limitation is imposed on the ML-236B non-producing microorganism provided that it is a cell to be transformed by the DNA vector, but examples include non-producing variants of the ML-236B producing microorganism. The recombinant DNA vector is presumed to have a desired function if the production of ML-236B is restored by the transformation to the variant.

The transformation method for functional expression is selected as needed, depending on the host cell. The

transformation of *Penicillium citrinum*, a preferred ML-236B producing microorganism, can be performed by preparing protoplasts from spores of *Penicillium citrinum*, and then introducing a recombinant DNA vector into the protoplast (as described in Nara, F. et al., Curr. Genet. 23. 28(1993)).

The protoplast is prepared in the following manner.

Spores from a slant on which *Penicillium citrinum* has been cultured are inoculated onto a plate of PGA agar medium and kept at 22 to 28°C for 10 to 14 days. The spores are collected from the plate and 1×10^7 to 1×10^9 spores are inoculated into 50 to 100 ml of a YPL-20 medium (composition: 0.1% (w/v) yeast extract (product of Difco corporation), 0.5% (w/v) polypeptone (product of Nihon Seiyaku corporation), 20% (w/v) of lactose, pH 5.0). The resulting medium is kept at 22 to 28°C for 18 hours to 2 days. The germinating spores are recovered from the culture, and treated with a cell wall lytic enzyme to yield a protoplast. No particular limitation is imposed on the cell wall lytic enzyme insofar as it can degrade the cell wall of *Penicillium citrinum* and does not adversely act on the microorganism. Examples include zymolyase, chitinase and the like.

The transformed ML-236B producing microorganism can be cultured under conditions suited for each host cell. When the transformant of *Penicillium citrinum* which is a preferable ML-236B producing microorganism is used, its cell wall is regenerated in advance by culturing the protoplast of the transformed microorganism under proper conditions.

The regeneration of the cell wall can be effected by sandwiching a VGS middle-layer agar medium (composition: Vogel minimum medium, 2% (w/v) glucose, 1M glucitol, 2% (w/v) agar), in which the protoplast of the transformed *Penicillium citrinum* has been enclosed therein, between a VGS lower-layer agar medium (composition: Vogel minimum medium, 2% (w/v) glucose, 1M glucitol, 2.7% (w/v) agar] and a VGS upper-layer agar medium (composition: Vogel minimum medium, 2% (w/v) glucose, 1M glucitol, 1.5% (w/v) agar) and then keeping the stack at 22 to 28°C for 7 to 15 days. The resultant strain is

subcultured on a PGA medium while keeping at 22 to 28°C. The resulting strain is inoculated, by using a platinum loop, to a slant prepared on a PGA medium, kept at 22 to 28°C for 10 to 14 days, and then stored at 0 to 4°C.

ML-236B can be efficiently produced by inoculating, from the slant on which the *Penicillium citrinum* transformant having a cell wall regenerated as described above has been cultured, the transformant into an MBG3-8 medium, and keeping the resulting medium at 22 to 28°C for 7 to 12 days while shaking. ML-236B can also be produced from *Penicillium citrinum* used as a host cell by employing an utterly same liquid culture.

ML-236B from the culture of ML-236B producing microorganism can be purified by using various techniques ordinarily employed for the purification of natural products in combination. No particular limitation is imposed on the technique and examples include centrifugal separation, solid-liquid separation by filtration, treatment with an alkali or acid, extraction with an organic solvent, dissolution while changing a solvent, chromatography such as adsorption chromatography, partition chromatography or the like, crystallization and the like. ML-236B is either in the hydroxy acid form or in the lactone form, which is interchangeable each other. Furthermore, the hydroxy acid forms a stable salt. By making use of such a physicochemical property, any one of the hydroxy acid of ML-236B (which will hereinafter be called "free hydroxy acid"), hydroxy acid salt of ML-236B (which will hereinafter be called "hydroxy acid salt") and lactone of ML236-B (which will hereinafter be called "lactone") is available.

The desired compound is available as a free hydroxy acid by subjecting the culture to alkaline hydrolysis under heating or at normal temperature, thereby ring-opening and converting it into the corresponding hydroxy acid salt, acidifying the reaction mixture, filtering the acidified mixture, and extracting the filtrate with an organic solvent immiscible with water. No particular limitation is imposed on the organic

solvent immiscible with water. Examples include aliphatic hydrocarbons such as hexane and heptane, aromatic hydrocarbons such as benzene and toluene, halogenated hydrocarbons such as methylene chloride and chloroform, ethers such as diethyl ether, esters such as ethyl formate and ethyl acetate, and mixed solvents of at least two of the above-described solvents.

The desired compound is available as a hydroxy acid salt by dissolving the free hydroxy acid in an aqueous solution of an alkaline metal salt such as sodium hydroxide.

Furthermore, the desired compound is available as lactone by the ring closure of the free hydroxy acid by dehydrating it in an organic solvent while heating or by another method.

The free hydroxy acid, hydroxy acid salt or lactone thus obtained can be purified and isolated by column chromatography or the like. Although no particular limitation is imposed on the carrier for column chromatography, examples include "Sephadex LH-20" (product of Pharmacia Corporation), "Diaion HP-20" (product of Mitsubishi Chemical Corporation), silica gel, reversed phase carrier and the like, of which C18 series carriers are preferred.

No particular limitation is imposed on the determination method of the amount of ML-236B insofar as it is ordinarily employed for the determination of the amount of organic compounds. Examples include reversed phase high performance liquid chromatography (which will hereinafter be called "reverse phase HPLC") and the like. Determination by the reverse phase HPLC can be carried out by subjecting the culture of an ML-236 producing microorganism to alkaline hydrolysis, subjecting the soluble fraction to reverse phase HPLC using a C18 column, measuring ultraviolet absorption and then quantifying the absorption value. The C18 column is not particularly limited insofar as it is ordinarily employed in the reverse phase HPLC. Examples include SSC-ODS-262 (product of Senshu Scientific Co., Ltd., diameter: 6 mm, length: 100 mm) and the like. The mobile phase is not particularly limited insofar as it is a solvent ordinarily employed for reverse

phase HPLC. Examples include 75% (v/v) methanol - 0.1% (v/v) triethylamine - 0.1% (v/v) acetic acid and the like. When ML-236B is added to an SSC-ODS-262 column at room temperature while using 75% (v/v) methanol - 0.1% (v/v) triethylamine - 0.1% (v/v) acetic acid as mobile phase at a rate of 2 ml/min, ML-236B is eluted after 4.0 minutes. ML-236B can be detected using a UV detector for HPLC. The absorption wavelength of the UV detector is 220 to 280 nm, preferably 220 to 260 nm, more preferably 236 nm.

A desired recombinant DNA vector having a gene whose functional expression has been confirmed is useful for improving the productivity of ML-236B.

In this specification, adenine, guanine, thymine and cytosine are described as "a", "g", "t" and "c", respectively. The base sequence indicated in each SEQ ID No. of the Sequence Listing is described in accordance with a guideline for preparing specification or the like including a base sequence or an amino acid sequence" (published by Japanese Patent Office, in June, 1998).

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating the construction of DNA vector pSAKcos1 which can be introduced into *Escherichia coli* and filamentous fungus and into which a long DNA can be inserted;

FIG. 2 is a diagram illustrating the analysis of a structural gene of the inserted sequence of pML 48; and

FIG. 3 is a diagram illustrating Northern blot hybridization of the inserted sequence of pML 48.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will hereinafter be described in further detail by Examples and Test. It should however be borne in mind that the present invention is not limited to or by them.

Example 1. Preparation of pSAKcos1 vector

1) Plasmid pSAK333 (Japanese Patent Application Publication No. Hei 3-262486) having a hygromycin B phosphotransferase gene originating from *Escherichia coli* was digested with restriction enzyme BamHI (product of Takara Shuzo Co., Ltd.), followed by treatment to form blunt ends with T4DNA polymerase (product of Takara Shuzo Co., Ltd).

2) The DNA fragment obtained above was self-ligated into a circular form by using "DNA ligation kit Ver.2" (product of Takara Shuzo Co., Ltd.), and competent cell JM109 strain of *Escherichia coli* (product of Takara Shuzo Co., Ltd.) was then transformed. A strain having a plasmid from which the BamHI site had been deleted was selected from the transformed *Escherichia coli*, and the plasmid possessed by this strain was designated as pSAK360.

3) After digestion of pSAK 360 with restriction enzyme PvuII, alkaline phosphatase treatment was conducted, whereby dephosphorylation at the 5'end was conducted. After a [SalI-ScaI] fragment (about 3kb) containing a cos site was obtained from a cosmid vector pWE15 (product of STRATAGENE GmbH) and was treated to form blunt ends with T4 DNA polymerase, it was ligated to the PvuII site of pSAK360, whereby the JM109 strain was transformed. The strain having a plasmid into which [SalI-ScaI] fragment (about 3kb) had been inserted at the PvuII site was selected from the transformed *Escherichia coli* and the plasmid of the strain was designated as pSAKcos1. The pSAKcos1 has a restriction enzyme recognition site of each of BamHI, EcoRI and NotI originating from pWE15. The pSAKcos1 has an ampicillin resistance gene and a hygromycin resistance gene as selection markers. In the below-described examples, when *Escherichia coli* was used as a host, a transformant by pSAKcos1 or transformant by pSAKcos1 to which a foreign gene had been inserted was selected by adding 40 μg/ml ampicillin to a medium. When *Penicillium citrinum* SANK13380 was used as a host, on the other hand, a transformant by pSAKcos1 or transformant by pSAKcos1 to which a foreign gene had been inserted was selected by adding 200 μg/ml hygromycin B to a medium.

The construction procedure of pSAKcos1 is shown in FIG. 1.

Example 2: Preparation of genomic DNA of *Penicillium citrinum* SANK13380 strain

1) Culturing of *Penicillium citrinum* SANK13380 strain

A seed culture of a *Penicillium citrinum* SANK13380 strain was made on a slant using a PGA agar medium. Described specifically, the *Penicillium citrinum* SANK13380 strain was inoculated by a platinum loop and kept at 26°C for 14 days. This slant was stored at 4°C.

Main culturing was performed by liquid aerobic culture. Cells of a 5 mm square of the above-mentioned slant were inoculated in a 500-ml Erlenmeyer flask containing 50 ml of an MBG3-8 medium, and shake-cultured under the conditions of 26°C and 210 rpm for 5 days.

2) Preparation of genomic DNA from the culture of *Penicillium citrinum* SANK 13380 strain

The culture obtained in 1) was centrifuged for 10 minutes under the conditions of room temperature and 1000 × G, and cells were collected. The resulting cells having a wet weight of 3 g were disrupted in a mortar cooled with dry ice until they took the powdery form. The cells thus disrupted were charged in a centrifuge tube filled with 20 ml of 62.5 mM EDTA·2Na (product of Wako Pure Chemical Industries, Ltd.) - 5% (w/v) SDS - 50 mM Tris (product of Wako Pure Chemical Industries, Ltd.) - hydrochloric acid (product of Wako Pure Chemical Industries, Ltd.) buffer (pH 8.0). After gentle mixing, the mixture was allowed to stand at 0°C for 1 hour. To the reaction mixture was added 10 ml of phenol saturated with 10 mM Tris - hydrochloric acid - 0.1mM EDTA·2Na (pH 8.0, which will hereinafter be called "TE"), followed by gentle mixing at 50°C for 1 hour. The reaction mixture was centrifuged for 10 minutes under the conditions of room temperature and 10000 × G and then, 15 ml of the upper layer (water phase) was poured in another centrifuge tube. After addition of 0.5 time by volume of TE saturated phenol and 0.5 time by volume of a chloroform

solution and gentle stirring for 2 minutes, the mixture was centrifuged for 10 minutes under the conditions of room temperature and 10000 × G (which will hereinafter be called "phenol-chloroform extraction"). To 10 ml of the upper layer (water phase) were added 10 ml of 8M ammonium acetate (pH 7.5) and 25 ml of 2-propanol (product of Wako Pure Chemical Industries, Ltd.). After cooling at -80°C for 15 minutes, the mixture was centrifuged for 10 minutes under the conditions of 4°C and 10000 × G. The precipitate was dissolved in 5 ml of TE. To the resulting solution were added 20 µl of 10 mg/ml "Ribonuclease A" (product of Sigma Chemical Co.) and 250 units of "Ribonuclease T1" (product of GIBCO BRL) and the mixture was kept at 37°C for 20 minutes. To the reaction mixture was added 20 ml of 2-propanol, and after gentle mixing, genomic DNA in the form of thread was spooled at the tip of a Pasteur pipette, and dissolved in 1 ml of TE. To the resulting DNA solution were added 0.1 time by volume of 3M sodium acetate (pH 6.5) and 2.5 times by volume of ethanol. After cooling at -80°C for 15 minutes, centrifugal separation was performed for 5 minutes under the conditions of 4°C and 10000 × G (which will hereinafter be called "ethanol precipitation"). The precipitate thus obtained was dissolved in 200 µl of TE. The resulting solution was used as a genomic DNA fraction.

Example 3: Preparation of genomic DNA library of *Penicillium citrinum* SANK13380 strain

1) Preparation of genomic DNA fragment

To 100 µl of an aqueous solution of genomic DNA (50 µg) of *Penicillium citrinum* SANK13380 strain obtained in Example 2 was added 0.25 unit of "Sau3AI" (product of Takara Shuzo Co., Ltd.). After the lapse of 10, 30, 60, 90 and 120 seconds, a 20 µl portion of the mixture was sampled, and 20 µm of 0.5M EDTA (pH 8.0) was added to each sample to terminate the restriction enzyme reaction. The resulting partially digested DNA fragments were separated by agarose gel electrophoresis, and agarose gel containing DNA fragments of 30 kb or greater was recovered.

The gel thus recovered was finely crushed, and charged in "Ultrafree C3 Centrifugal Filter device" (manufactured by Nihon Millipore KK). After the gel was cooled and frozen at -80°C for 15 minutes, it was thawed by keeping at 37°C for 10 minutes and centrifuged at 5000 × G for 5 minutes, whereby a DNA extract was obtained. The resulting DNA extract was subjected to phenol-chloroform extraction and ethanol precipitation. The precipitate was then dissolved in a small amount of TE.

2) Pretreatment of DNA vector pSAKcos1

After pSAKcos1 was digested with restriction enzyme BamHI (product of Takara Shuzo Co., Ltd.), alkaline phosphatase (product of Takara Shuzo Co., Ltd.) reaction was effected at 65°C for 30 minutes. The reaction mixture was then subjected to phenol-chloroform extraction and ethanol precipitation and the precipitate thus obtained was dissolved in a small amount of TE.

3) Ligation and in vitro packaging

The genomic DNA fragment (2 μg) as described above in 1) and the pretreated pSAKcos1 (1 μg) as described above in 2) were mixed. Using a DNA ligation kit Ver. 2 (product of Takara Shuzo Co., Ltd.), the resulting mixture was ligated at 16°C for 16 hours. The reaction mixture was subjected to phenol-chloroform extraction and ethanol precipitation and the precipitate thus obtained was dissolved in 5 μl of TE. The solution containing the ligation product was subjected to in vitro packaging using "GIGAPAK II Gold kit" (product of STRATAGENE GmbH), whereby transformed *Escherichia coli* containing a recombinant DNA vector was obtained. On a plate on which colonies of the transformed *Escherichia coli* had been formed, 3 mL of an LB medium was poured and then, the colonies were recovered from the plate using a cell scraper (which will hereinafter be called "recovered solution 1"). The plate was washed with another 3 ml portion of the LB medium, and colonies were recovered (which will hereinafter be called "recovered solution 2"). A mixture of the recovered solutions 1 and 2 was added with glycerin to give a final concentration

of 18%. The resulting solution was called *Escherichia coli* cell solution and was stored at -80°C as a genomic DNA library of *Penicillium citrinum* SANK 13380 strain.

Example 4: Amplification of PKS gene fragment by PCR using genomic DNA of *Penicillium citrinum* SANK13380 strain as a template

1) Design and synthesis of PCR primer.

Based on the amino acid sequence of the PKS gene of *Aspergillus flavus* (as described in Brown, D.W., et al., Proc. Natl. Acad. Sci. USA, 93, 1418(1996)), mix primers shown in SEQ ID Nos. 3 and 4 of the Sequence Listing were designed and synthesized.

SEQ ID No. 3 of the Sequence Listing: gayacngcntgyasttc

SEQ ID No. 4 of the Sequence Listing: tcnccnknrcwgtgncc

In the base sequence of SEQ ID Nos. 3 and 4 of the Sequence Listing, n represents an inosine base (hypoxanthine), y represents t or c, s represents g or c, k represents g or t, r represents g or a, and w represents a or t.

2) Amplification of DNA fragment by PCR

A liquid (50 μl) containing the PCR primers as described above in 2) (each 100 pmol), the genomic DNA of *Penicillium citrinum* SANK13380 strain obtained in Example 2 (500 ng), 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 50 mM potassium chloride, 2 mM magnesium chloride and 1.25 units of "Ex. Taq DNA polymerase" (product of Takara Shuzo Co., Ltd.) was subjected to a cyclic reaction comprising three continuous steps, that is, 1 minute at 94°C, 2 minutes at 58°C and 3 minutes at 70°C. The cycle was repeated 30 times to amplify the DNA fragment. PCR was performed using "TaKaRa PCR Thermal Cycler MP TP3000" (product of Takara Shuzo Co., Ltd.).

The DNA fragment thus amplified was subjected to agarose gel electrophoresis, and then agarose gel containing the DNA fragment having a size of from about 1.0 to 2.0 kb was recovered. DNA was recovered from the gel, and subjected to phenol-chloroform extraction and ethanol precipitation. The precipitate was dissolved in a small amount of TE.

3) Ligation and transformation

By using "TA cloning system pCR2.1" (product of Invitrogen Corporation), the DNA fragment obtained in 2) was ligated to the plasmid pCR2.1 included in the kit, whereby a transformant was obtained.

Several clones were selected, and cultured according to the method of Maniatis, et al., (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Described specifically, each of the colonies was inoculated into a 24-ml test tube containing 2 ml of an LB medium, and was shake cultured at 37°C for 18 hours.

A recombinant DNA vector was prepared from the resulting culture according to the alkaline method (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)). Described specifically, 1.5 ml of the culture solution was centrifuged for 2 minutes under the conditions of room temperature and 10000 × G and from the precipitate thus obtained, cells were recovered. To the cells, was added 100 µl of 50 mM glucose - 25 mM Tris - hydrochloric acid - 10 mM EDTA (pH 8.0) to suspend the former in the latter. To the resulting suspension was added 200 µl of 0.2N sodium hydroxide - 1% (w/v) SDS and the mixture was stirred gently to lyse the microorganism. The protein was then denatured by adding 150 µl of 3M potassium acetate - 11.5% (w/v) glacial acetic acid, followed by centrifugation for 10 minutes under the conditions of room temperature and 10000 × G. The supernatant was recovered, and then subjected to phenol-chloroform extraction and ethanol precipitation. The precipitate thus obtained was dissolved in 50 µl of TE containing 40 µg/ml of "Ribonuclease A" (product of Sigma Chemical Co.).

Each of the recombinant DNA vectors was digested with an restriction enzyme, and subjected to electrophoresis. The inserted base sequences, in the recombinant DNA vectors, having different electrophoresis patterns were determined using a DNA sequencer (Model 377: product of Perkin Elmer

Japan).

As a result, existence of a strain having a recombinant DNA vector containing a PKS gene fragment was confirmed.

Example 5: Genomic Southern blotting hybridization of *Penicillium citrinum* SANK13380 strain

1) Electrophoresis and transfer to membrane

The genomic DNA (10 μg) of *Penicillium citrinum* SANK13380 strain obtained in Example 2 was digested with restriction enzymes EcoRI, SalI, HindIII or Sac1 (each, product of Takara Shuzo Co., Ltd.), and then subjected to agarose gel electrophoresis. For the preparation of the agarose gel, Agarose L03 "TAKARA" (product of Takara Shuzo Co., Ltd.) was employed. After electrophoresis, the gel was immersed in 0.25N hydrochloric acid (product of Wako Pure Chemical Industries, Ltd.) and was gently shaken at room temperature for 10 minutes. The gel was transferred into 0.4N sodium hydroxide (product of Wako Pure Chemical Industries, Ltd.), and gently shaken at room temperature for 30 minutes. According to the alkaline transfer method of Maniatis, et al. (as described in Maniatis, T., et al., Molecular cloning, a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989)), DNA in the gel was transferred to a nylon membrane Hybond™-N+ (product of Amersham Biosciences), and immobilized thereon. The membrane was washed with 2 × SSC (composition of 1 × SSC: 150 mM NaCl and 15 mM trisodium citrate), followed by air drying.

2) Hybridization and detection of signal

The membrane obtained in 1) was subjected to hybridization with the PKS gene fragment obtained in Example 4 as a probe.

As the probe, the PKS gene fragment DNA (1 μg) obtained in Example 4 was labeled by "DIG DNA Labelling Kit" (product of Boehringer Mannheim) and just before use, was quenched after 10 minutes boiling.

The membrane described in 1) was immersed in a hybridization liquid ("DIG Easy Hyb", product of Boehringer

Mannheim). After pre-hybridization at 42°C for 2 hours while shaking at 20 rpm, the above-described labeled probe was added to the hybridization liquid, and hybridization was performed at 42°C for 18 hours while shaking at 20 rpm in a "Multishaker Oven HB" (product of TAITEC). After the hybridization, the membrane was washed three times with 2 × SSC at room temperature for 20 minutes, and then twice with 0.1 × SSC at 55°C for 30 minutes.

The membrane thus washed was treated with "DIG Luminescent Detection Kit for Nucleic Acids" (product of Boehringer Mannheim) and exposed to an X ray film ("Lumifilm"; product of Boehringer Mannheim). Exposure was performed using "Fuji Medical Film Processor FPM 800A" (product of Fuji Photo Film Co., Ltd.).

As a result, it was confirmed that the PKS gene fragment obtained in Example 4 existed on the genome of *Penicillium citrinum*.

Example 6: Screening of genomic DNA library of *Penicillium citrinum* SANK13380 strain using PKS gene fragment as a probe

The genomic DNA containing a PKS gene was cloned by the colony hybridization method.

1) Preparation of membrane

The *Escherichia coli* cell solution stored as a genomic DNA library of *Penicillium citrinum* SANK13380 strain (as described in Example 3) was diluted and then spread on an LB agar medium plate so that 5000 to 10000 colonies per plate would grow. After the plate was kept at 26°C for 18 hours, it was cooled at 4°C for 1 hour. On the plate was placed "Hybond™-N+" (product of Amersham Biosciences) and they were brought into contact for 1 minute. The membrane having the colonies adhered thereon was carefully separated from the plate. With the surface side in contact with the colonies up, the membrane was immersed in 200 ml of 1.5M sodium chloride - 0.5N sodium hydroxide for 7 minutes, and then immersed twice in 200 ml of 1.5M sodium chloride - 0.5M Tris-hydrochloric acid - 1 mM EDTA (pH 7.5) for 3 minutes, followed by washing

with 400 ml of 2 × SSC. The membrane thus washed was air-dried for 30 minutes.

2) Hybridization

As the probe, the PKS gene fragment DNA (1 μg) obtained in Example 4 was labeled by "DIG DNA Labelling Kit" (product of Boehringer Mannheim) and just before use, was quenched after 10 minutes boiling.

The membrane described in 1) was immersed in a hybridization liquid ("DIG Easy Hyb", product of Boehringer Mannheim). After pre-hybridization at 42°C for 2 hours while shaking at 20 rpm, the above-described labeled probe was added to the hybridization liquid, and hybridization was performed at 42°C for 18 hours while shaking at 20 rpm in a "Multishaker Oven HB" (product of TAITEC). After the hybridization, the membrane was washed three times with 2 × SSC at room temperature for 20 minutes, and then twice with 0.1 × SSC at 68°C for 30 minutes.

The membrane thus washed was treated with "DIG Luminescent Detection Kit for Nucleic Acids" (product of Boehringer Mannheim) and exposed to an X ray film ("Lumifilm"; product of Boehringer Mannheim). Exposure was performed using "Fuji Medical Film Processor FPM 800A" (product of Fuji Photo Film Co., Ltd.).

The operations as described in 1) and 2) are called "screening".

A peripheral portion of the colony of the clone from which a positive signal had been detected upon the first screening was scraped off and suspended in an LB medium. The resulting suspension was spread onto a plate after dilution as needed, and cultured. Second screening was performed in a similar manner, whereby the positive clone was purified.

The positive clone obtained in the present example, that is, transformed *Escherichia coli* pML48 SANK71199 strain was internationally deposited with the Research Institute of Life Science and Technology, the Agency of Industrial Science and Technology, Ministry of International Trade and Industry (1-1-

3, Higashi, Tsukuba, Ibaraki, Japan) as of July 7, 1999 and accorded accession No. FERM BP-6780.

Example 7: Analysis of the inserted sequence of recombinant DNA vector pML48 (1)

Culturing of the *Escherichia coli* pML48 SANK71199 strain obtained in Example 6 and preparation of a recombinant DNA vector from the culture were performed in accordance with the method as described in Example 4.

The recombinant DNA vector thus obtained was designated as pML48. The inserted sequence of pML48 was digested with various restriction enzymes, and resulting fragments were subcloned by incorporating them in pUC119 (product of Takara Shuzo Co., Ltd.). With the subclones thus obtained as probes, Southern blot hybridization was performed in accordance with the method as described in Example 5. Described specifically, the restriction enzyme digests of pML48 were each subjected to electrophoresis, and the DNA transferred to a membrane was subjected to hybridization.

As a result, a restriction enzyme map of the inserted sequence of pML48 was made.

The base sequence of the inserted sequence of each of the subclones was determined using "DNA Sequencer Model 377" (product of Perkin Elmer Japan Co. Ltd.), whereby the entire base sequence of pML48 was determined.

The inserted sequence of pML48 consisted of 34203 bases in total.

The base sequence of the inserted sequence of pML48 is described in SEQ ID Nos. 1 and 2 of the Sequence Listing. The base sequences described in SEQ ID Nos. 1 and 2 of the Sequence Listing are completely complementary each other.

Existence of a structural gene on the inserted sequence was analyzed using a gene searching program GRAIL ("ApoCom GRAIL Toolkit": product of APOCOM Corporation) and a homology searching program BLAST ("Gapped-BLAST (BLAST2)" included in WISCONSIN GCG package ver. 10.0).

As a result, existence of six different structural genes

in the inserted base sequence of pML48 was predicted, and they were designated as mlcA, mlcB, mlcC, mlcD, mlcE and mlcR respectively. It was predicted further that mlcA, mlcB, mlcE and mlcR each has a coding region in the base sequence of SEQ ID NO. 2 of the Sequence Listing, while mlcC and mlcD each has a coding region in the base sequence of SEQ ID NO. 1 of the Sequence Listing. The relative position and length of each of the predicted structure genes of the inserted sequence were also predicted.

The results of this example are shown in FIG. 2.

Example 8: Analysis of the inserted sequence of recombinant DNA vector pML48 (2)

Expression analysis of the structural genes whose existence had been suggested in Example 7 and analysis of 5'- and 3'-end regions were carried out by the Northern Blot Hybridization method and RACE.

1) Preparation of total RNA of *Penicillium citrinum* SANK13380

Cells from a slant of 5 mm square on which *Penicillium citrinum* SANK13380 strain had been cultured (as described in Example 2) were inoculated in a 100-ml Erlenmeyer flask containing 10 ml of an MGB3-8 medium, and shake-cultured at 26°C for 3 days.

Total RNA was prepared from the culture by using "RNeasy Plant Mini Kit" (product of Qiagen AG) adopting the guanidine - isothiocyanate method. Described specifically, the culture was centrifuged for 10 minutes under the conditions of room temperature and 5000 × G and cells were recovered. After the cells having 2 g of wet weight were frozen with liquid nitrogen, they were disrupted into powder on a mortar. The resulting powder was suspended in 4 ml of a lytic buffer (included in the kit) containing guanidine isothiocyanate. In 10 QIAshredder spin columns included in the kit, 450 µl portions of the resulting suspension were dispensed, followed by centrifugal separation at room temperature and 1000 × G for 10 minutes, whereby eluates were recovered, respectively. A 225 µl portion of ethanol was added to each eluate and then the

resulting mixture was added to RNA mini spin column included in the kit. After the column was washed with a washing buffer included in the kit, the adsorbate was caused to elute using a 50 µl portion of ribonuclease-free distilled water. The resulting eluate was used as total RNA fraction.

2) Northern blot hybridization

An RNA sample was prepared by adding, to 2.25 µl of an aqueous solution containing 20 µg of total RNA of *Penicillium citrinum* SANK13380, 1 µl of 10 × MOPS (product of Dojindo Laboratories, composition: 200 mM 3-morpholinopropanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA·2Na; pH 7.0; used after autoclave sterilization at 121°C for 20 minutes), 1.75 µl of formaldehyde and 5 µl of formamide, followed by mixing. The RNA sample thus obtained was kept at 65°C for 10 minutes, quenched in ice water, and then, subjected to agarose gel electrophoresis. The gel for electrophoresis was prepared by mixing 10 ml of 10 × MOPS and 1 g of Agarose L03 "TAKARA" (product of Takara Shuzo Co., Ltd.) with 72 ml of diethyl pyrocarbonate (product of Sigma Chemical Co.) treating water, heating the resulting mixture to dissolve the agarose therein, cooling, and adding 18 ml of formaldehyde to the solution. As the sample buffer, 1 × MOPS (prepared by diluting 10 × MOPS to 10 times with water) was used. RNA in the gel was transferred to Hybond™-N+ (product of Amersham Biosciences) in 10 × SSC.

As the probe, DNA fragments (a, b, c, d and e) available by digesting the pML48 inserted sequence with the restriction enzymes 1 and 2 as shown in the following Table 1 were used.

Table 1: Probe for Northern blot hybridization

Probe	Restriction Enzyme 1	Nucleotide No. of Restriction Enzyme Recognition Site*	Restriction Enzyme 2	Nucleotide No. of Restriction Enzyme Recognition Site *
a	EcoRI	6319 to 6324	EcoRI	15799 to 15804
b	BamHI	16793 to 16798	PstI	18164 to 18169
c	KpnI	26025 to 26030	BamHI	27413 to 27418
d	SalI	28691 to 28696	SalI	29551 to 29556
e	HindIII	33050 to 33055	SacI	34039 to 34044

* Each nucleotide No. is based on SEQ ID No. 1 of the Sequence

Listing

Labeling of probes, hybridization and detection of signals were performed in accordance with the Southern blot hybridization in Example 5.

The results of the present Example are shown in FIG. 3.

Each signal shows the existence of a transcription product homologous to the base sequence of each probe.

It has been confirmed that in this Example, mlcB, mlcD, mlcE and mlcR, among the 6 structural genes predicted to exist on the pML48 inserted sequence, were transcribed in the *Penicillium citrinum* SANK13380 strain and transcription of mlcA and mlcC in the cell was also suggested.

The position of each signal does not indicate the relative size of the transcript.

3) Determination of 5'-end sequence according to 5'RACE

Acquisition of cDNA containing the 5'-end region of each structural gene was carried out using "5' RACE System for Rapid Amplification of cDNA ends, Version 2.0" (product of GIBCO BRL).

In each structural gene on the pML48 inserted sequence predicted from the results of Example 7 and 2) of this Example, two kinds of antisense-side oligo DNAs were designed based on the base sequence presumed to exist in the coding region in the vicinity of the 5'-end of each structural gene.

The base sequence of the antisense-side oligo DNA (1) designed based on the base sequence on the 3'-end side of each structural gene was shown in Table 2, while the base sequence of the antisense-side oligo DNA (2) designed based on the base sequence on the 5'-end side of the each structural gene was shown in Table 3.

Table 2: Oligo DNA (1) used for 5'-end sequence analysis by 5' RACE

Gene	SEQ ID No. of the Sequence Listing: Base Sequence
mlcA	SEQ ID No. 5: gcatgttcaatttgctctc
mlcB	SEQ ID No. 6: ctggatcagacttttctgc

mlcC	SEQ ID No. 7: gtcgcagtagcatgggcc
mlcD	SEQ ID No. 8: gtcagagtgatgctcttctc
mlcE	SEQ ID No. 9: gttgagaggattgtgagggc
mlcR	SEQ ID No. 10: ttgcttgtgttggattgtc

Table 3: Oligo DNA (2) used for 5'-end sequence analysis by 5' RACE

Gene	SEQ ID No. of the Sequence Listing: Base Sequence
mlcA	SEQ ID No. 11: catggtactctcgcccgttc
mlcB	SEQ ID No. 12: ctccccagtacgtaagctc
mlcC	SEQ ID No. 13: ccataatgagtgtgactgttc
mlcD	SEQ ID No. 14: gaacatctgcatccccgtc
mlcE	SEQ ID No. 15: ggaaggcaaagaaagtgtac
mlcR	SEQ ID No. 16: agattcattgctgttggcatc

The first strand of cDNA was synthesized via reverse transcription using the oligo DNA (1) as a primer and the total RNA of *Penicillium citrinum* SANK 13380 strain as a template. Described specifically, 24 μl of a reaction mixture containing 1 μg of total RNA, 2.5 pmol of oligo DNA (1) and 1 μl of SUPER SCRIPTTM II reverse transcriptase (included in the kit) was kept at 16°C for 1 hour, and the reaction product was added to "GLASSMAX spin cartridge" included in the kit, whereby the first strand of cDNA strand was purified.

To the 3'-end of the first strand of cDNA was added a poly C chain by using terminal deoxyribonucleotidyl transferase included in the kit.

A reaction mixture (50 μl) containing the first strand of cDNA having a poly C chain added at the 3'-end, 40 pmol of oligo DNA (2) and 40 pmol of Abriged Anchor Primer (included in the kit) was kept at 94°C for two minutes. The reaction mixture was then kept at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 2 minutes, and with this reaction as one cycle, the reaction was repeated 35 times. Then, the reaction mixture was kept at 72°C for 5 minute and 4°C for 18 hours. After the resulting product was subjected to agarose gel electrophoresis, DNA was recovered from the gel. The product was purified by phenol-chloroform extraction and ethanol precipitation, and cloned using pCR 2.1 in accordance with the method as described in Example 4.

The above-described operation is called "5' RACE".

The base sequence of the cDNA fragment containing 5'-end was determined, and transcription initiation point and the position of translation initiation codon were predicted.

The SEQ ID No. of the Sequence Listing in which the base sequence of the 5'-end cDNA fragment corresponding to each structural gene obtained by 5' RACE is shown in Table 4. In Table 5, described are SEQ ID No. in which the transcription initiation point and translation initiation point of each structural gene exist, the position of the translation initiation point and the position of the translation initiation point.

Table 4: SEQ ID Nos of the Sequence Listing in which the base sequence of 5'-end cDNA fragment is shown

Gene	SEQ ID No. of Sequence Listing
mlcA	SEQ ID No. 17
mlcB	SEQ ID No. 18
mlcC	SEQ ID No. 19
mlcD	SEQ ID No. 20
mlcE	SEQ ID No. 21
mlcR	SEQ ID No. 22

Table 5: Positions of transcription initiation point and translation initiation codon

Gene No.	SEQ ID No. in which translation initiation codon exists *	Nucleotide in SEQ ID No. 1 or 2	
		Transcription initiation point	Translation initiation codon
mlcA	SEQ ID No. 2	22913	23045 to 23047
mlcB	SEQ ID No. 2	11689	11748 to 11750
mlcC	SEQ ID No. 1	11641	11796 to 11798
mlcD	SEQ ID No. 1	24066	24321 to 24323
mlcE	SEQ ID No. 2	3399	3545 to 3547
mlcR	SEQ ID No. 2	365	400 to 402

* Base sequences shown in SEQ ID Nos. 1 and 2 of Sequence Listing are completely complementary each other.

4) Determination of 3'-end sequence by 3' RACE

Acquisition of cDNA containing the 3'-end region of each structural gene was carried out using "Ready To Go: T-Primed First-Strand kit" (product of Pharmacia corporation).

In each structural gene on the pML48 inserted base sequence predicted from the results of Example 7 and 2) of this Example, one kind of sense-side oligo DNA (3) presumed to exist in a coding region in the vicinity of the 3'-end was prepared. The base sequence of the oligo DNA (3) prepared for each structural gene is shown in Table 6.

Table 6: Oligo DNA (3) used for 3'-end sequence analysis by 3'RACE

Gene	SEQ ID No. of Sequence Listing : Base Sequence
mlcA	SEQ ID No. 23: atcataccatcttcaacaac
mlcB	SEQ ID No. 24: gctagaataggttacaagcc
mlcC	SEQ ID No. 25: acattgccaggcacccagac
mlcD	SEQ ID No. 26: caacgcccaagctgccaatc
mlcE	SEQ ID No. 27: gtcttttcctactatctacc
mlcR	SEQ ID No. 28: ctttcccagctgctactatc

The first strand of cDNA was synthesized via reverse transcription using oligo DNA (3) as a primer and total RNA (1 μg) of *Penicillium citrinum* SANK13380 strain as a template.

A reaction mixture (100 μl) containing the first strand of cDNA, 40 pmol of oligo DNA (3) and NotI-d(T) 18 primer (included in the kit) was kept at 94°C for 2 minutes. It was then kept at 94°C for 30 seconds, at 55°C for 30 seconds and at 72°C for 2 minutes, and with this reaction as one cycle, the reaction was repeated 35 times. The mixture was then kept at 72°C for 5 minutes and at 4°C for 18 hours. The resulting product was subjected to agarose gel electrophoresis. From the gel, DNA was recovered. The product was purified by phenol-chloroform extraction and ethanol precipitation, and cloned in accordance with the method described in Example 4 by using pCR 2.1.

The above-described operation is called 3'-RACE.

The base sequence of the 3'-side fragment of the cDNA thus obtained was determined, and the position of the translation termination codon was predicted.

SEQ ID Nos. of the Sequence Listing in which the base sequence of the 3'-end cDNA fragment corresponding to each structural gene obtained by 3' RACE are listed in Table 7. In

Table 8, the translation termination codon of each structural gene and position of the codon are described based on SEQ ID No.1 or 2 of the Sequence Listing.

Table 7: SEQ ID Nos of the Sequence Listing in which the base sequence of 3'-end cDNA fragment are shown

Gene	SEQ ID No. of Sequence Listing
mlcA	SEQ ID No. 29
mlcB	SEQ ID No. 30
mlcC	SEQ ID No. 31
mlcD	SEQ ID No. 32
mlcE	SEQ ID No. 33
mlcR	SEQ ID No. 34

Table 8: Translation termination codon of each structural gene and the position of the translation termination codon

Gene	Translation termination codon	SEQ ID No. in which translation termination codon exists	Nucleotide No. of translation termination codon in SEQ ID No. 1 or 2
mlcA	tag	SEQ ID No. 2	32723 to 32725
mlcB	taa	SEQ ID No. 2	19840 to 19842
mlcC	taa	SEQ ID No. 1	13479 to 13481
mlcD	tga	SEQ ID No. 1	27890 to 27892
mlcE	tga	SEQ ID No. 2	5730 to 5732
mlcR	tag	SEQ IE No. 2	1915 to 1917

* Base sequence shown in SEQ ID Nos. 1 and 2 of the Sequence Listing are completely complementary each other.

The C-terminal amino acid residue of the polypeptide predicted to be encoded by each structural gene, the base sequence of the trinucleotide encoding the amino acid residue and the position of the trinucleotide are shown in Table 9.

Table 9: C-terminal amino acid residue of polypeptide encoded by each structural gene

Gene	C-terminal amino acid residue	Base sequence of trinucleotide encoding the amino acid	SEQ ID No. in which the trinucleotide exists *	Nucleotide No. of the trinucleotide in SEQ ID No. 1 or 2

mlcA	Alanine	gcc	SEQ ID No. 2	32720 to 32722
mlcB	Serine	agt	SEQ ID No. 2	19837 to 19839
mlcC	Cysteine	tgc	SEQ ID No. 1	13476 to 13478
mlcD	Arginine	cgc	SEQ ID No. 1	27887 to 27889
mlcE	Alanine	gct	SEQ ID No. 2	5727 to 5729
mlcR	Alanine	gct	SEQ IE No. 2	1912 to 1914

* The base sequences shown in SEQ ID No.1 and 2 of Sequence Listing are completely complementary each other.

The sequence complementary to the translation termination codon as shown in Table 8, the SEQ ID No. in which the complementary sequence exists and the position of the complementary sequence are listed in Table 10.

Table 10 Sequence complementary to translation termination codon of each structural gene

Gene	Sequence complementary to translation termination codon	SEQ ID No. in which the complementary sequence exists *	Nucleotide No. of the complementary sequence in SEQ ID No. 1 or 2
mlcA	cta	SEQ ID No. 1	1479 to 1481
mlcB	tta	SEQ ID No. 1	14362 to 14364
mlcC	tta	SEQ ID No. 2	20723 to 20725
mlcD	tca	SEQ ID No. 2	6312 to 6314
mlcE	tca	SEQ ID No. 1	28472 to 28474
mlcR	cta	SEQ IE No. 1	32287 to 32289

* The base sequences shown in SEQ ID No.1 and 2 of the Sequence Listing are completely complementary each other.

As described above, existence, direction and position of each structural gene were revealed. Based on these data, it is possible to acquire the transcription and translation products of each structural gene.

Example 9: Transformation of *Penicillium citrinum* by using recombinant DNA vector pML48

The transformation of *Penicillium citrinum* was carried out in accordance with the method of Nara, et al. (as described in Nara, F., et al., Curr. Genet. 23, 28(1993))

1) Preparation of protoplasts

From a slant on which the *Penicillium citrinum* SANK

13380 strain had been cultured, spores were inoculated onto a PGA agar medium by using a platinum loop and they were kept at 26°C for 14 days. Of the spores of the *Penicillium citrinum* SANK 13380 strain recovered from the culture, 1×10^8 spores were inoculated onto 80 ml of an YPL-20 medium and kept at 26°C for 1 day. After confirmation of the germination of the spores by microscopic observation, the germinating spores were centrifuged for 10 minutes under the conditions of room temperature and 5000 × G. Thus, the spores were recovered as a precipitate. After washing three times with sterilized water, the spores were converted into protoplasts. Described specifically, 200 mg of "Zymolyase 20T" (product of Seikagaku Corporation) and 100 mg of chitinase (product of Sigma Chemical Co.) were dissolved in 10 ml of 0.55M magnesium chloride, and centrifuged for 10 minutes under the conditions of room temperature and 5000 × G. The supernatant thus obtained was used as an enzyme solution. In a 100-ml Erlenmeyer flask were charged 20 ml of the enzyme solution and 0.5 g, in wet weight, of germinating spores and they were shaken gently at 30°C for 60 minutes. After confirmation of the conversion of the germinating spores into protoplasts by microscopic observation, the reaction mixture was filtered through a "3G-2 glass filter" (product of HARIO Co., Ltd.). The filtrate was centrifuged for 10 minutes under the conditions of room temperature and 1000 × G, and the protoplasts were recovered as a precipitate.

2) Transformation

The protoplasts obtained in 1) were washed twice with 30 ml of a 0.55M magnesium chloride solution and once with 30 ml of a solution of 0.55M magnesium chloride - 50 mM calcium chloride - 10 mM 3-morpholinopropanesulfonic acid (which will hereinafter be called "MCM solution", pH 6.3), and then suspended in 100 µl of 4% (w/v) polyethylene glycol 8000 - 10 mM 3-morpholinopropane sulfonic acid - 0.0025% (w/v) heparin (product of Sigma Chemical Co.) - 50 mM magnesium chloride (which will hereinafter be called "solution for transformation", pH 6.3). The solution for transformation (96

μl) containing about 5×10^7 protoplasts was mixed with 10 μl of TE containing 120 μg of pML48 DNA. The mixture was allowed to stand on ice for 30 minutes. To the reaction mixture was added 1.2 ml of 20% (w/v) polyethylene glycol - 50 mM magnesium chloride - 10 mM 3-morpholinopropanesulfonic acid (pH 6.3), followed by gentle pipetting. The mixture was allowed to stand at room temperature for 20 minutes. An MCM solution (10 ml) was then added. After gentle mixing, the resulting mixture was centrifuged for 10 minutes under the conditions of room temperature and 1000 × G. The transformed protoplasts were recovered from the precipitate.

3) Regeneration of the cell wall of transformed protoplasts

The transformed protoplasts obtained in 2) were suspended in 5 ml of a liquid VGS middle-layer agar medium. The resulting suspension was stacked over 10 ml of a solidified VGS lower-layer agar medium plate. After culturing at 26°C for 1 day, 10 ml, per plate, of a liquid VGS upper-layer agar medium containing 5 mg of "Hygromycin B" (product of Sigma Chemical Co., Ltd.) was stacked (to give a final concentration of "Hygromycin B" of 200 μg/ml) over the plate. The strain obtained by keeping the culture at 26°C for 14 days was subcultured on a PGA agar medium containing 200 μg/ml of Hygromicin B, subcultured on a slant prepared using a PGA agar medium, and kept at 26°C for 14 days.

The strain thus obtained is called "transformed strain". The slant was stored at 4°C.

Test 1: Comparison in ML-236B producing ability between transformed strain and parent strain

The transformed strains obtained in Example 9 and the *Penicillium citrinum* SANK 13380 strain, that is, a parent strain were cultured and the amount of ML-236B in each culture was measured.

A 5 mm square inoculum from the slant (as described in Example 9) in which the transformed strain had been cultured or from the slant (as described in Example 2) in which the *Penicillium citrinum* SANK 13380 strain had been cultured was

inoculated in a 100-ml Erlenmeyer flask containing 10 ml of an MBG3-8 medium and shake-cultured at 26°C for 2 days. After addition of 3.5 ml of a 50% (w/v) glycerin solution, shake culture was continued at 26°C for 10 days.

To 10 ml of the culture was added 50 ml of 0.2N sodium hydroxide. The resulting mixture was kept at 26°C for 1 hour while shaking, followed by centrifugal separation for 2 minutes under the conditions of room temperature and 3000 × G. The supernatant (1 ml) was recovered and it was mixed with 9 ml of 50% methanol. The resulting mixture was provided for HPLC.

Elution was conducted at room temperature at a flow rate of 2 ml/min while using "SSC-ODS-262" (diameter: 6 mm and length: 100 mm; product of Senshu Kagaku Co., Ltd.) as a column for HPLC, and 75% (v/v) methanol - 0.1% (v/v) triethylamine - 0.1% (v/v) acetic acid as a mobile phase. Under these conditions, elution of ML-236B was detected 4.0 minutes after the addition to the column. Detection was performed by a UV detector whose absorption wavelength was set at 236 nm.

Of the transformed strains, five strains were improved in ML236b production ability and their production ability was higher by 12% on average than that of the parent strain.

The ML-236B producing ability of these five strains was maintained stably even after subculture such as monospore treatment.

[Industrial Applicability]

In the present invention, DNA obtained from the ML-236B producing microorganism improves the ML-236B producing ability of the producing microorganism by being introduced therein.

In addition, existence, direction and position of 6 structural genes on the DNA have been revealed. The present invention makes it possible to obtain cDNA corresponding to each structural gene.